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(54) Title: SPONGIFORM ENCEPHALOPATHY DETECTION METHODS

(57) Abstract

A method for detecting the presence of a spongiform encephalopathy (e.g. Bovine Spongiform Encephalopathy) in an animal comprising determining the presence and/or amount of agent (e.g. by 2D-polyacrylamide gel electrophoresis and staining) in a body fluid (e.g. cerebrospinal fluid) of the animal which has a molecular weight of between 35-39 kDa, and a pI of 5.3 and which does not cross-react with antibody raised against apoliprotein E and relating the result of this determination to a control value (e.g. from a known uninfected animal) so as to be able to detect the likely presence of spongiform encephalopathy in the animal.

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SPONGIFORM ENCEPHALOPATHY DETECTION METHODS

The present invention relates to methods for the detection of spongiform encephalopathies in animals, and in particular to the detection of bovine spongiform encephalopathy (BSE) in cattle.

Spongiform encephalopathies are a class of diseases which include scrapie in sheep and Creutzfeldt-Jakob disease in humans. BSE is a notifiable fatal neurodegenerative disease found in cattle. BSE is of major importance to the British farming industry.

Currently cases of BSE are identified by clinical manifestations in the animal. Cases are confirmed by post-mortem analysis of brain tissue, for instance by histopathology, by detection of scrapie associated fibrils or proteinase K resistant protein.

These methods have the disadvantage that they necessitate the slaughter of potentially-infected animals which may turn out to be disease-free. Alternatively, clinical signs may be absent or go undetected, thus leaving infected animals in the herd.

Thus there exists a need for a pre-mortem test for BSE which can be used when diagnosing potentially-infected animals.

The present invention has now provided a method for detecting spongiform encephalopathy in an animal which addresses some, and in preferred forms all, of these problems.

According to one aspect of the present invention there is provided a method for detecting the presence of a spongiform encephalopathy in an animal comprising determining the presence and/or amount of agent in a body fluid of the animal which has a molecular weight of between 35-39 kDa, a pI of approximately 5.3, and which does not cross-react with antibody raised against apoliprotein E and relating the result of this determination to the infection status of the animal.

Preferably the result of the determination is compared with a control

value and the relationship between the two is correlated with the infection status of the animal.

Preferably the method is used to detect BSE.

Thus the discovery that the spongiform encephalopathy infection in an animal may be correlated with the presence of, or an increase in the concentration of, an agent or agents in the body fluids of that animal forms the basis for the methods of the current invention.

Apolipoprotein E is cholesterol transporting protein produced in the peripheral and central nervous system. Its presence in either multiple- or single-forms has been categorised in cerebrospinal fluid (CSF) and serum. Its molecular weight (37 kDa) and pI (around 5.4 to 5.7) are similar to those of the agent described above. However the agent which is determined in the methods of the present invention does not cross-react with anti-apolipoprotein E antibody.

It should be noted that there is no requirement to accurately determine the agent concentration because the spongiform encephalopathy infection status of the animal may be detected by comparison with a control.

The control value may be derived from the agent concentration in a different animal (for which the infection status is known) and which is analysed in parallel with the test animal. Alternatively, the control value may come from the same animal, or be a known standard.

In either case the control value may be determined using the same method used for the test animal or using a different analytical method.

The results from the 'control' animal may be used to derive a standard control value, or to calibrate the test animal result.

Preferably the body fluid analysed in the method is CSF since the proximity of the CSF to brain means that neurological disorders which

produce alterations in the protein composition of the brain may be manifested in the CSF. Methods for extracting samples of CSF are well known to those skilled in the art.

The invention embraces any method for determining the presence and/or amount of agent in a body fluid of an animal which is currently comprised in the art, and any methods which may later become available.

Preferably the presence and/or amount of agent in a body fluid of the animal is derived by the use of polyacrylamide gel electrophoresis (PAGE) to separate out the agents having both a molecular weight of between 35-39 kDa and a pI of approximately 5.3 from other materials in the body fluid, and then staining the gel and making densitometry measurements in the region of the gel containing the agent so as to determine the presence and/or amount of the agent present.

Preferably the PAGE is two dimensional polyacrylamide gel electrophoresis (2DPAGE) and the stain is silver stain.

Preferably the lack of cross reactivity between the agent and anti-apolipoprotein E antibody agent is confirmed by the use of antibody raised against apolipoprotein E. Immunogen-based techniques for measuring cross-reactivity are well known to those skilled in the art eg. ELISA or Western Blotting.

In alternative embodiments of the invention, these immunogenic techniques may be used both to identify the agent and to estimate its concentration. This can be achieved by raising antibodies against the agent, or a synthetic peptide derived from the sequence thereof.

Thus the invention makes available methods for detecting the presence of spongiform encephalopathy in a test animal which address many, and in preferred forms all, of the problems of the prior art. The balance between test certainty and ease of use will be dependent on the precise method of agent analysis chosen for use in the methods of the current invention. However, the pre-mortem diagnosis of BSE in

cattle opens up the possibility of mass-testing in herds, thereby reducing the likelihood of slaughtering uninfected animals or 'missing' infected ones.

The methods of the present invention will now be described, by way of illustration only, through reference to the following example and figures. Other embodiments falling within the scope of the invention will occur to those skilled in the art in the light of this.

EXAMPLE - IDENTIFICATION OF BSE IN CATTLE

Sample preparation: CSF samples were collected from BSE-positive cattle and BSE-negative cattle. In each case the diagnosis was confirmed by post-mortem histopathology and electron microscopy. CSF samples were taken by cisternamagna puncture after death and concentrated 10-15 fold. Volumes of CSF containing 40 µg total protein were mixed in a 4:1 ratio with denaturing solution (1g sodium dodecyl sulphate (SDS) and 0.232g dithiothreitol in 10ml water) and heated at 95°C for 5 minutes. Samples were then pulse centrifuged.

Electrophoresis: The prepared samples were 2D electrophoresed using a Millipore Investigator 2D electrophoresis system according to the method in the instruction manual. First dimensional iso-electric-focussing was carried out in 26 cm threaded glass tubes with 1 mm inner diameter in a pH gradient of 3-10 for 18000 volt hours after pre-focussing the gels for 1 hour to 1500 V. Second dimension SDS-PAGE was carried out using 1 mm thick large format gels (23 cm x 23 cm) with 12.5% acrylamide and no stacking gel.

Staining and Image analysis: The 2D gels were silver stained according to the Millipore manual. Gels were scanned with an Omnimedia scanner XRS and analysed using Bioimage software and Investigator Database programme (Millipore) using a sunSPARC station computer.

Confirmation of the identity of the agent: The 2D gels were electroblotted onto Immobilon-P membranes overnight at 30V using a

Bio-Rad Trans-Blot cell. The blots were blocked using Tween 80 for 1 hour and then incubated for 90 minutes with sheep antiserum containing polyclonal antibody raised against apolipoprotein E. Bound sheep antibodies were detected using rabbit anti-sheep IgG and a horseradish peroxidase detection system.

Comparison of BSE-negative and BSE-positive cattle: A comparison of the stained gels from typical BSE-positive and -negative samples is shown in Fig 1(a) and Fig 1(b). As can be seen the number and intensity of the silver stained spots in the region corresponding to agents having an approximate molecular weight of 34-38 kDa, and a pI of around 5.4 - 5.7 (labelled 'Apo E') is higher in the BSE-positive sample. Those spots in the region labelled 'Apo E' were found to cross react with anti-apolipoprotein E antibody. However, the 2-3 spots, molecular weight 35-39 kDa and pI 5.3 (arrowed in each gel), corresponding to one or more agents which did not cross react with this antibody, are undetectable in the BSE-negative sample but are present in clearly detectable amounts in the BSE-positive sample

Using a number of samples of body fluid from different cattle samples it was found that the agent was consistently present in detectable amounts in BSE-positive animals but was undetectable in BSE-negative animals, thus indicating that the presence and/or amount of this agent may be used to detect the likely presence of BSE in potentially infected animals.

CLAIMS

1. A method for detecting the presence of a spongiform encephalopathy in an animal comprising determining the presence and/or amount of agent in a body fluid of the animal which has a molecular weight of between 35 and 39 kDa, a pI of approximately 5.3, and which does not cross-react with antibody raised against apoliprotein E and relating the result of the determination to the infection status of the animal.

2. A method as claimed in claim 1 wherein the result of the determination is compared with a control value and the relationship between the two is correlated with the infection state of the animal.

3. A method as claimed in claim 1 or claim 2 wherein the spongiform encephalopathy is bovine spongiform encephalopathy.

4. A method as claimed in any one of the preceding claims wherein the body fluid analysed in the method is cerebrospinal fluid.

5. A method as claimed in any one of the preceding claims wherein the presence and/or amount of agent in a body fluid of the animal is derived by the use polyacrylamide gel electrophoresis (PAGE) to separate out the agents having both a molecular weight of between 35 and 39 kDa and a pI of 5.3 from other materials in the body fluid, and then staining the gel and making densitometry measurements in the region of the gel containing the agent so as to determine the presence and/or amount of the agent present.

6. A method as claimed in claim 5 wherein the PAGE is two dimensional polyacrylamide gel electrophoresis.

7. A method as claimed in claim 5 or claim 6 wherein the stain is silver stain.

8. A method as claimed in any preceding claim wherein the lack of cross reactivety between the agent and anti-apolipoprotein E

antibody is confirmed by the use of antibody raised against apolipoprotein E.

9. A method as claimed in any of claims 1 to 4 wherein antibodies are raised against the agent, or a synthetic peptide derived generated from the sequence thereof, and the presence and/or amount of agent in a body fluid of the animal is determined by use of these antibodies.

10. A method as claimed in any one of claims 2 to 9 wherein the control value is derived by the same method as that used with the animal but using a further animal for which the infection status is known.

11. A method for detecting the presence of spongiform encephalopathy in an animal substantially as described hereinbefore.

Fig.1(b).

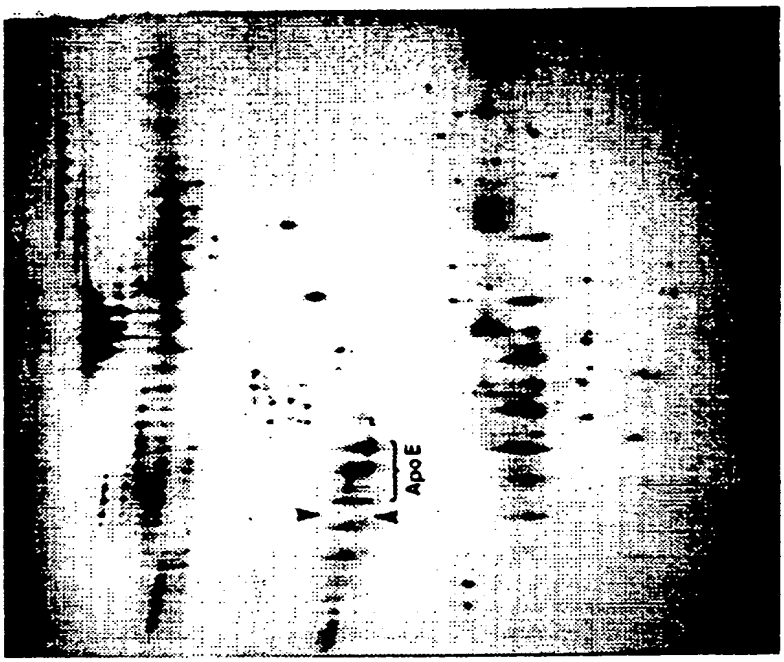
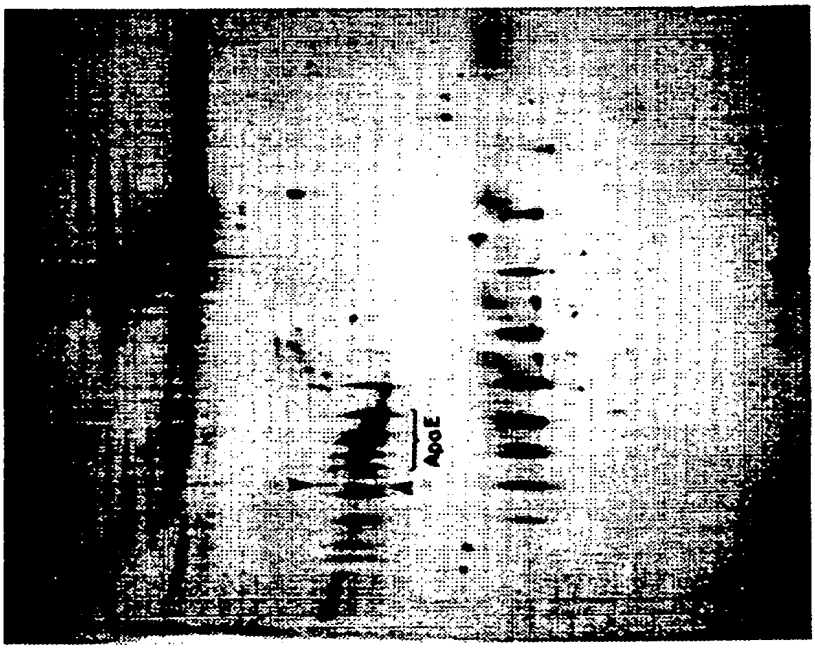


Fig.1(a).



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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A,4 892 814 (M.G. HARRINGTON ET AL.) 9 January 1990 see the whole document --- -/--	1-11

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 120, no. 25, 20 June 1994 Columbus, Ohio, US; abstract no. 320660v, page 634; column 2; see abstract & SHINKEI KENKYU NO SHINPO, vol. 37, no. 6, - 1993 TOKYO, pages 1039-1051, Y. NAMBA 'Immunochemical demonstration of apolipoprotein E in cerebral amyloid deposits in Alzheimer's disease and kuru plaque amyloids in Creutzfeldt-Jacob disease.' see the whole document	1-11
A	NATURE, vol. 336, no. 6197, 24 November 1988 LONDON UK, pages 390-392, XP 000566424 J. HOPE ET AL. 'Fibrils from brains of cows with new cattle disease contain scrapie-associated protein.' see figures 2,3	1-11
A	NEW ENGLAND JOURNAL OF MEDICINE, vol. 315, no. 2, 31 July 1986 BOSTON MA USA, pages 279-283, XP 000567332 M.G. HARRINGTON ET AL. 'Abnormal proteins in the cerebrospinal fluid of patients with Creutzfeldt-Jacob disease.' cited in the application see the whole document	1-11

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Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4892814	09-01-90	NONE	